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(54) Title: REPROGRAMMING OF SOMATIC CELL NUCLEI

(57) **Abstract:** The invention provides methods for cloning mammals that allow the donor chromosomes to be reprogrammed prior to insertion into an enucleated oocyte. The invention also features methods of inserting chromosomes or nuclei into recipient cells.

REPROGRAMMING OF SOMATIC CELL NUCLEI

This application is being filed on 20 January 2004 as a PCT International Patent application in the name of the University of Massachusetts, a US national corporation, applicant for the designation of all countries except the US, and James M. Robl, a U.S. citizen, Philippe Collas a citizen of France, Pedro Moreira, a citizen of Portugal, and Jacqueline Deruyter, a U.S. citizen, applicants for the designation of the US only.

FIELD OF THE INVENTION

This invention relates generally to improved methods for cloning mammals and mammalian cells, and methods for inserting chromosomes, nuclei, or condensed chromatin into recipient cells.

BACKGROUND OF THE INVENTION

The cloning of non-human mammals allows the production of multiple animals with an identical nuclear DNA content. The donor genetic material used to generate these mammals may be selected from a pool of animals based upon a particular phenotype such as the quantity of milk production or it can be genetically modified to provide animals with enhanced properties for a wide array of applications such as increased resistance to disease, recombinant protein production in milk, and xenotransplantation. Unfortunately, the efficiency of cloning mammals using donor somatic cells is generally low, resulting in only about 1-5% of nuclear transplant embryos developing to term (Polejaeva et al, Nature 407:86-90, 2000).

A significant problem with cloning is the loss of mid to late term pregnancies and the low viability of some offspring (Cibelli et al., Nature Biotech. 20:13-14, 2002). Cloning also has application in the reprogramming of somatic cells to an embryonic state for the purpose of deriving cell-based therapies. More efficient methods are needed for cloning mammals and mammalian cells. Improved methods are necessary to reduce the cost and time required to generate multiple viable non-human offspring. In addition, nuclear transfer techniques that produce fewer adverse effects in the recipient cells are desired. Therefore, a heretofore-unaddressed need exists in the art to address the aforementioned deficiencies and inadequacies.

SUMMARY OF THE INVENTION

The purpose of the present invention is to provide improved methods for cloning non-human mammals and mammalian cells. In particular, these methods

involve the condensation of a donor nucleus into condensed chromatin to allow the release of nuclear components such as transcription factors and factors that modulate the structure of chromatin that otherwise may promote the transcription of genes that are undesirable for the development of the nuclear transplant embryo into a viable non-human offspring or for reprogramming mammalian somatic cells into cells with an alternate state of differentiation such as an embryonic cell. In a related method, the plasma membrane of a donor cell is removed, breached, or otherwise compromised so as to make it porous, such that the genome of said cell is incubated with a reprogramming media (e.g., a cell extract) to allow the addition or removal of factors from the genome of the cell, and then the genome is transferred into an enucleated oocyte to generate a cloned embryo. If desired, the steps of any of these methods may be repeated one or more times or different reprogramming methods may be performed sequentially to increase the extent of reprogramming, resulting in greater viability of the cloned fetuses.

The invention also provides methods for generating chimeric embryos in which some or all of the placental tissue is from one genetic source and the majority of the fetal tissue is from another genetic source. These chimeric embryos may have fewer placental abnormalities and thus may have an increased survival rate. In addition, a novel method has been developed for the insertion of condensed chromatin or a nucleus into the recipient oocyte that involves the use of fusigenic compounds.

Accordingly, in a first aspect, the invention provides a method of cloning a non-human mammal. This method involves (a) isolating donor nuclei, (b) incubating a donor nucleus under conditions that allow formation of a condensed chromatin without causing DNA replication, (c) inserting the condensed chromatin into an enucleated oocyte, thereby forming a nuclear transfer oocyte, and (d) transferring the nuclear transfer oocyte or an embryo formed from the nuclear transfer oocyte into the uterus of a host mammal under conditions that allow the nuclear transfer oocyte or embryo to develop into a fetus. It is desirable for the donor nucleus to be contacted with one or more of the following under conditions that allow formation of condensed chromatin: a mitotic extract in the presence or absence of an anti-NuMA antibody, a detergent and salt solution, or a protein kinase solution.

In one preferred embodiment, the reprogramming media (e.g., a cell extract) is modified by the enrichment or depletion of a factor, such as a DNA methyltransferase, histone deacetylase, histone, nuclear lamin, transcription factor, activator, or repressor. In other aspects, the level of expression of NuMA or

AKAP95 protein in the oocyte is greater in the nucleus than in the cytoplasm. Still in other aspects, a portion of the AKAP95 protein in the oocyte is extracted.

In another preferred embodiment, the condensed chromatin is purified from the extract prior to insertion into the nucleated oocyte. In yet another preferred embodiment, inserting the condensed chromatin into the enucleated oocyte involves contacting the condensed chromatin and the oocyte with a fusigenic compound under conditions that allow the condensed chromatin to enter the oocyte. In yet another preferred embodiment, the non-human fetus develops into a viable offspring. Preferably, at least 3, 5, 10, 20, 30, 40, 50, 60, 70, 80, or 90% of the oocytes develop into viable offspring. In this method, the oocyte containing the condensed chromatin may be cultured under conditions that allow cell division and one of the resulting cells may be recloned one or more times. The donor nucleus and the oocyte used in the method may be from the same species, or they may be from different species or genuses. The mammal may be a human or non-human animal, and the oocyte may be fertilized or unfertilized. In the case where the resulting cloned embryo is used to derive embryonic cells for research and/or therapy, the species may include human. Preferably the donor nucleus is from a G_1 or G_0 phase cell. In addition, the genomic DNA of the cloned embryo, fetus, or mammal is preferably substantially identical to that of the donor cell. It is also contemplated that the condensed chromatin may be inserted into an embryo for the production of a chimeric embryo, non-human fetus, or non-human mammal containing a mixture of cells with DNA substantially identical to that of the condensed chromatin and cells with DNA substantially identical to that of the naturally-occurring cells in the embryo. It is also contemplated that a nucleated oocyte may be used in the methods of the invention.

The invention also provides methods of inserting chromosomes or nuclei into recipient cells. These methods are useful for transferring donor genetic material into a recipient oocyte for the cloning of a non-human mammal. These methods may also be used to replace the genetic material of one cell with that of another cell.

According to this aspect of the invention, a technique is provided for inserting chromosomes into a recipient cell that involves contacting the chromosomes and the cell with a fusigenic compound under conditions that allow the chromosomes to enter the recipient cell. In one preferred embodiment, the chromosomes are incubated with the fusigenic compound prior to being contacted with the recipient cell. The chromosomes may be condensed or not condensed, and the chromosomes and the recipient cell may be from the same species or may be from different species or genuses. In another preferred embodiment, the recipient cell is a fertilized or unfertilized oocyte. Preferably, the recipient cell or the

chromosomes are from a human or non-human mammal. In various embodiments, the recipient cell is an adult, fetal, or embryonic cell. In one particular preferred embodiment, all of the chromosomes of a donor cell are inserted into the recipient cell. The donor cell can be haploid (DNA content of n), diploid (2n), or tetraploid (4n), and the recipient cell can be hypodiploid (DNA content of less than 2n), haploid, or enucleated. In another embodiment, the chromosomes are from more than one donor cell, such as two haploid cells. In yet another preferred embodiment, the chromosomes are obtained by contacting a donor nucleus that has less than four sets of homologous chromosomes with a mitotic extract, a detergent and salt, or a protein kinase under conditions that allow formation of condensed chromatin without causing DNA replication.

Useful fusigenic compounds include polyethylene glycol (PEG), and lipids such as Lipofectin®, Lipofectamin®, DOTAP® {N-[I-(2,3-Dioleoyloxy)propyl]-N, N, N-trimethylamonium methylsulfate; $C_{43}H_{83}NO_8S$}, DOSPA® {2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N, N dimethyl-l -propanaminium trifuoroacetate }, and DOPE® (dioleoyl phosphatidylethanolamine). Other useful lipids include neutral and monovalent or multivalent cationic lipids, such as those containing quaternary ammonium groups. Additional lipids have a cholesterol moiety such as that formed from the reaction of the hydroxyl group in cholesterol with a group in the lipid. Still other lipids have a saturated or unsaturated fatty acid that can contain between 5 and 10, 10 and 15, 15 and 20, or 20 and 30 carbon atoms, inclusive. These lipids may be synthesized using standard chemical synthesis techniques, obtained from naturally-occurring sources, or purchased from commercially available source (Summers *et al.*, Biophys J. 71(6):3199-206, 1996; Nabekura *et al.*, Pharm Res. 13(7):1069-72, 1996; Walter *et al.*, Biophys J. 66(2 Pt 1):366-376, 1994; Yang et al., Biosci Rep. 13(3):143-157, 1993; Walter and Siegel, Biochemistry; 6: 32(13):3271-3281, 1993).

Other useful fusigenic compounds are phospholipids such as membrane vesicle fractions from sea urchin eggs or any other source (Collas and Poccia, J. of Cell Science 109, 1275:1283, 1996). Preferably, contacting chromosomes with the membrane vesicle fraction does not result in the chromosomes being encapsulated by an intact membrane.

In a related aspect, the invention provides a method of inserting a nucleus into a recipient cell that includes contacting the nucleus and the cell with a fusigenic compound under conditions that allow the nucleus to enter the recipient cell. The fusigenic compound is either a lipid or is not a polymer consisting of identical monomers. Preferably, the nucleus is incubated with the fusigenic compound prior to being contacted with the recipient cell. In various embodiments, the nucleus and

the recipient cell are from the same species or are from different species or different genuses. The nucleus can be haploid, diploid, or tetraploid, and the recipient cell can be hypodiploid, haploid, or enucleated. In one embodiment, the recipient cell is a fertilized or unfertilized oocyte. Preferably, the recipient cell or the nucleus is from a human or a non-human mammal. In other embodiments, the recipient cell is an adult, fetal, or embryonic cell. Useful fusigenic compounds are lipids such as Lipofectin®, Lipofectamin®, DOTAP®, DOSPA®, and DOPE®. Other lipids include neutral lipids and monovalent or multivalent cationic lipids, such as those containing quaternary ammonium groups. Additional lipids have a cholesterol moiety or a saturated or unsaturated fatty acid that preferably contains between 5 and 10, 10 and 15, 15 and 20, or 20 and 30 carbon atoms, inclusive. Other fusigenic compounds are phospholipids such as membrane vesicle fractions from sea urchin eggs or any other source (Collas and Poccia, *supra*). Preferably, contacting a nucleus with the membrane vesicle fraction does not result in the nucleus being encapsulated by an intact membrane.

In certain embodiments of various aspects of the invention, the nucleus or chromosomes are from an adult, fetal, or embryonic cell. The nucleus or chromosomes may also be obtained from any of the following preferred donor cells, or they may be inserted into any of the following preferred recipient cells. Examples of preferred cells include differentiated cells such as epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melauocytes, chondrocytes, B-lymphocytes, T-lymphocytes, erythrocytes, macrophages, monocytes, fibroblasts, and muscle cells; and undifferentiated cells such as embryonic stem cells and embryonic germ cells. In another embodiment, the cell is from the female reproductive system, such as a mammary gland, ovarian cumulus, grandulosa, or oviductal cell. Preferred cells also include those from any organ, such as the bladder, brain, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver, lung, ovaries, pancreas, prostate, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, and uterus. Preferred non-human mammals include members of the genus Bos. Examples of other preferred mammals include cows, sheep, big-horn sheep, goats, buffalos, antelopes, oxen, horses, donkeys, mule, deer, elk, caribou, water buffalo, camels, llama, alpaca, rabbits, pigs, mice, rats, guinea pigs, cats, dogs, hamsters, and primates such as monkeys. In yet another embodiment, the nucleus or chromosomes are from a transgenic cell or mammal or contain a mutation not found in the donor cell or not found in a naturally-occurring cell.

Useful transgenic donor nuclei encode proteins that confer improved resistance to disease or parasites in the cloned mammal. Alternatively, the donor

nuclei may be engineered so that the cloned mammal produces a recombinant product, such as the production of a human protein in the urine, blood, or milk of a bovine. For example, proteins may be expressed in the urine of cattle by inserting a polynucleotide sequence encoding a human protein under the control of an uroplakin promoter. Examples of therapeutic proteins that may be produced in the milk of cloned bovines include human monoclonal antibodies and human clotting factors such as any of factors I to XIII (Voet and Voet, Biochemistry, John Wiley& Sons, New York, 1990). These heterologous proteins may be expressed under the control of a prolactin promoter or any other promoter suitable for expression in the milk of a bovine. For the production of human antibodies in the milk, blood, or other fluids of cloned mammals, standard methods may be used to inactivate or "knock out" the endogenous genes for antibody heavy or light chains so that functional antibodies are no longer encoded by a donor nucleus and to insert genes encoding the heavy and light chains of human IgA, IgD, IgE, IgG, or IgM into the genome of the donor nucleus. Recombinant proteins from these or other tissues or fluids may be purified using standard purification methods (see, for example, Ausubel et al., *supra*).

It is also contemplated that cells, tissues, or organs from an embryo, fetus, or adult mammal produced using the methods of the invention may be used as a source of material for medical applications, such as the treatment or prevention of disease in humans. For example, cells, tissues, or organs may be developed *in vitro* from cloned embryonic cells and then transferred to a mammal (e.g., a human), removed from a cloned non-human mammal and transferred to another mammal of a different species, or removed from a cloned non-human mammal and transferred to another non-human mammal of the same species. For example, neuronal tissue from a cloned non-human mammal or cloned mammalian embryo may be grafted into an appropriate area in the human nervous system to treat, prevent, or stabilize a neurological disease such as Alzheimer's disease, Parkinson's disease, Huntington's disease, ALS, retinitis pigmentosum, age-related macular degeneration, or a spinal cord injury. In particular, degenerating or injured neuronal cells may be replaced by the corresponding cells from a cloned non-human mammal or mammalian embryo. This transplantation method may also be used to treat, prevent, or stabilize diseases including, but not limited to, blood cell disorders including disorders of the immune system such as autoimmune diseases, rheumatoid arthritis, pemphigus vulgaris, multiple sclerosis, myasthenia gravis, anemias, blood cell cancers, or defects in hematopoiesis associated with radiation or chemotherapy, disorders of the vascular system such as tumor angiogenesis, wound healing, stroke, and heart failure, diabetes, indeed any disease treatable by means of cell-based therapy. In these procedures, the cells that are attacked by the recipient's own immune system may be

replaced by transplanted cells. The cloned mammals may also be used as a source of cartilage, bone marrow, or any other tissue or organ.

For the production of a cloned non-human mammal or mammalian embryo as a source of donor transplant material, the donor nucleus used to generate the cloned mammal is preferably modified to encode a heterologous MHC Class 1 protein having an amino acid sequence substantially identical to the sequence of an MHC Class 1 protein found in the recipient mammal that will be administered the donor material. Alternatively, the donor nucleus encodes a heterologous MHC Class 1 protein having an amino acid sequence substantially identical to the sequence of an MHC Class 1 protein found in another mammal of the same genus or species as the recipient mammal. These donor cells, tissues, or organs from cloned mammals that express heterologous MHC proteins are less likely to elicit an adverse immune response when administered to the recipient mammal. Other preferred donor transplant material is obtained from a cloned mammal that was generated using a donor nucleus which was modified to express a heterologous protein that inhibits the complement pathway of the recipient mammal, such as the human complement inhibitor CD59 or the human complement regulator decay accelerating factor (h-DAF) (see, for example, Ramirez et al., Transplantation 15:989-998,200; Costa et al., Xenotransplantation 6:6-16, 1999).

In yet another preferred embodiment, the donor nucleus has a mutation that reduces or eliminates the expression or activity of a galactosyltransferase, such as alpha(1,3) galactosyltransferase (Tearle et al., Transplantation 61:13-19, 1996; Sandrin, Immunol. Rev. 141:169-190, 1994; Costa et al., Xenotransplantation 6: 6-16, 1999). This enzyme modifies cell surface molecules with a carbohydrate that elicits an adverse immune response when cells expressing this galactose alpha-(1,3)-galactose epitope are administered to humans. Thus, donor transplant material that has a lower level of expression of this epitope may have a lower incidence of rejection by the recipient mammal.

The present invention provides a number of advantages related to the cloning of non-human mammals and mammalian embryos and the transfer of genomic material into recipient cells. For example, the methods may result in a higher percentage of viable offspring, increasing the number of mammals that may be used for agricultural or medical applications. Compared to microinjection, the method described herein for the transfer of chromosomes or nuclei into cells, called lipofusion, is a gentler and simpler means of introducing genetic material into cells since it does not require physical disruption of cellular structures or the technical skill needed to pick up a nucleus or condensed chromatin using a micropipette and inject it into a cell. The present methods may also be safer than fusion methods

involving viruses or viral components. Further, lipofusion is believed to elicit minimal, if any, physiological damage to the recipient cell and is therefore beneficial over electrofusion which elicits signaling events inside the fused cells that may impair cell cycle progression or development of the cloned embryo.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

DETAILED DESCRIPTION OF THE INVENTION

The novel methods of cloning mammals described herein involve remodeling of the donor genetic material before it is inserted into the recipient oocyte. Remodeling refers to any morphological change that improves development of the resulting nuclear transplant oocyte over that derived from either transferring whole cells or intact nuclei into a recipient oocyte. Reprogramming is achieved by incubating a donor nucleus in a mitotic extract, detergent and salt solution, or protein kinase solution, resulting in nuclear envelope dissolution and possibly chromatin condensation. This nuclear envelope breakdown and chromatin condensation allows the release of transcription regulatory proteins that were attached to the chromosomes and that would otherwise promote the transcription of genes undesirable for oocyte, embryo, or fetus development. Additional regulatory proteins may be removed by purifying the condensed chromatin prior to transferring it into a recipient oocyte. Alternatively, specific regulatory proteins that are released from the chromosomes may be immunodepleted or otherwise removed from the extract to prevent them from rebinding the chromosomes. After nuclear transfer, new proteins from the oocyte cytoplasm may be bound to the chromosomes during decondensation of the chromatin and nuclear envelope formation in the oocyte. These proteins promote the transcription of genes that allow the oocyte to develop into a viable offspring.

An additional embodiment of the present invention provides a method of cloning mammalian pluripotent stem cells. This method involves (a) incubating a donor nucleus at any stage of the cell cycle, including G_1, S, G_2, M, G_0, senescence, checkpoint arrested, cells arrested with compounds that inhibit progression through the cell cycle, nonproliferating differentiated cells, or unsynchronized proliferating cells under conditions that allow formation of condensed chromatin without causing DNA replication, (b) inserting the condensed chromatin into an enucleated oocyte, thereby forming a nuclear transfer oocyte, (c) incubating the nuclear transfer oocyte or an embryo formed from the nuclear transfer oocyte to allow development to proceed to the morula or blastocyst stage, and (d) deriving pluripotent stem cells

from the embryo including embryonic stem cells and human embryo-derived cells from which cells useful in cell therapy can be derived.

In another embodiment, incubating a permeabilized cell, condensed chromatin, or nucleus in the reprogramming media alters a phenotype of the permeabilized cell or a cell containing the reprogrammed condensed chromatin or nucleus relative to the phenotype of the donor cell. In yet another embodiment, incubating a permeabilized cell, condensed chromatin, or nucleus in the reprogramming media causes the permeabilized cell or a cell containing the reprogrammed condensed chromatin or nucleus to gain or lose an activity relative to the donor cell.

In certain embodiments, the donor nucleus is incubated with a reprogramming media (e.g., a cell extract) under conditions that allow nuclear or cytoplasmic components such as transcription factors, repressor proteins, or chromatin remodeling proteins to be added to, or removed from, the nucleus or resulting condensed chromatin. The donor nucleus can be contacted with one or more of the following under conditions that allow formation of condensed chromatin: a mitotic extract in the presence or absence of an anti-NuMA antibody, a detergent and/or salt solution, or a protein kinase solution. In other embodiments, the reconstituted oocyte or the resulting embryo expresses lamin A, lamin C, or NuMA protein at a level that is less than 5 fold greater than the corresponding level expressed by a control oocyte or a control embryo with the same number of cells and from the same species.

In a related aspect, the invention provides a method of cloning mammalian embryonic cells. This method involves (a) isolating a donor nucleus, (b) incubating a donor nucleus under conditions that allow formation of condensed chromatin without causing DNA replication, (c) inserting the condensed chromatin into an enucleated oocyte, thereby forming a nuclear transfer oocyte, (d) incubating the nuclear transfer oocyte or an embryo formed from the nuclear transfer oocyte until a compacting morulae or blastocyst is obtained, and (e) deriving embryonic stem cells or embryo-derived cells with a high degree of pluripotency useful in therapy and medical research.

In an additional aspect, the invention provides another method of cloning a non-human mammal or mammalian stem cells. This method involves incubating a permeabilized cell with a reprogramming media (e.g., a cell extract) under conditions that allow the removal of a factor (e.g., a nuclear or cytoplasmic component such as a transcription factor or chromatin-modifying activities) from a nucleus, condensed chromatin, or chromosome of the permeabilized cell or the addition of a factor to the nucleus, condensed chromatin, or chromosome, thereby

forming a reprogrammed cell. The reprogrammed cell is inserted into an enucleated oocyte, and the resulting oocyte or an embryo formed from the oocyte is transferred into the uterus of a host non-human mammal under conditions that allow the oocyte or embryo to develop into a fetus or the mammalian embryo can be used to derive embryonic stem cells for use in research and therapy.

Examples of cells that may be used to generate reprogramming extracts include, but are not limited to, embryonic stem cells, embryo-derived cells, and adult stem cells from brain, blood, bone marrow, pancreas, liver, skin, or any other organ or tissue. Other exemplary reprogramming cell extracts include, but are not limited to, oocyte extracts (e.g., bovine, frog, or sea urchin oocyte extracts) and male germ cell extracts (e.g., spermatogonia, spermatocyte, spermatid, or sperm extracts from vertebrates, invertebrates, or mammals such as bovine). The donor or permeabilized cell can be genetically unmodified, or it may be modified by the random integration of DNA, the addition of artificial chromosomes, or the targeted modification by homologous recombination. The donor cell, permeabilized cell, recipient cell, or cytoplast can be from a source of any age, such as an embryo, fetus, youth, or adult mammal and can be in any differentiated state.

In another aspect, the invention provides another method of cloning mammalian pluripotent stem cells. This method involves incubating a permeabilized mammalian cell with a reprogramming media (e.g., a cell extract) under conditions that allow the removal of a factor (e.g., a nuclear or cytoplasmic component such as a transcription factor) from a nucleus, condensed chromatin, or chromosome of the permeabilized cell or the addition of a factor to the nucleus, condensed chromatin, or chromosome, thereby forming a reprogrammed cell. The reprogrammed cell is inserted into an enucleated oocyte, and the resulting oocyte or an embryo formed from the oocyte is incubated in media that allows the development of the embryo to the point of a compacting morula or blastocyest, and cells such as embryonic stem cells or embryo-derived cells are obtained that are useful in cell therapy.

In a particular embodiment, the permeabilized cell is contacted with one or more of the following under conditions that allow formation of condensed chromatin: a mitotic extract in the presence or absence of an anti-NuMA antibody, a detergent and/or salt solution, or a protein kinase solution. In yet another preferred embodiment, the permeabilized cell is incubated with an interphase reprogramming media (e.g., an interphase cell extract). In still another embodiment, the nucleus in the permeabilized cell remains membrane-bounded, and the chromosomes in the nucleus do not condense during incubation with this interphase reprogramming media.

In other embodiments, incubating the permeabilized cell in the reprogramming media does not cause DNA replication or only causes DNA replication in less than 50, 40, 30, 20, 10, or 5% of the cells. Alternatively, incubating the permeabilized cell in the reprogramming media causes DNA replication in at least 60, 70, 80, 90, 95, or 100% of the cells. In a preferred embodiment, the permeabilized cell is formed by incubating an intact cell with a detergent, such as digitonin, or a bacterial toxin, such as Streptolysin O. In yet another embodiment, the reprogrammed cell is incubated under conditions that allow the membrane of the reprogrammed cell to reseal prior to insertion into the oocyte.

In another embodiment, the reconstituted oocyte or the resulting embryo expresses lamin A, lamin C, or NuMA protein at a level that is less than 5 fold greater than the corresponding level expressed by a control oocyte or a control embryo with the same number of cells and from the same species.

The invention also features a novel method, denoted lipofusion, for inserting a nucleus or chromosomes into cells. This method involves incubating the nucleus or chromosomes and the recipient cell with a fusigenic compound that allows the nucleus or chromosomes to be transferred into the cytoplasm of the cell. This method may generally be applied to nuclei and chromosomes from all cell types and to recipient cells of all cell types.

Without intent to limit the scope of the invention, these and other methods relevant to the present invention are described further below. Titles are used in the examples for convenience of a reader, which in no way should limit the scope of the invention. Moreover, certain theories are proposed and disclosed herein; however, in no way are they intended nor should they, whether they are right or wrong, limit the scope of the invention so long as data are processed, sampled, converted, or the like according to the invention without regard for any particular theory or scheme of action.

Definitions

As used herein, by "condensed chromatin" is meant more than one chromosome not enclosed by a membrane. Preferably, the condensed chromatin contains all of the chromosomes of a cell. Condensed chromatin contains condensed chromosomes that may be formed by exposure of a nucleus to a mitotic extract as described herein. Alternatively, condensed chromatin containing decondensed or partially condensed chromosomes may be generated by exposure of a nucleus to one of the following, as described herein: a mitotic extract containing an anti-NuMA antibody, a detergent and salt solution, or a protein kinase solution.

If desired, the level of chromosome condensation may be determined using standard methods by measuring the intensity of staining with the DNA stain, DAPI. As chromosomes condense, this staining intensity increases. Thus, the staining intensity of the chromosomes may be compared to the staining intensity for decondensed chromosomes in interphase (designated 0% condensed) and maximally condensed chromosomes in mitosis (designated 100% condensed). Based on this comparison, the percent of maximal condensation may be determined. Preferred condensed chromatin are at least 50, 60, 70, 80, 90, *or* 100% condensed. Preferred decondensed or partially condensed chromatin is less than 50, 40, 30, 20, or 10% condensed.

By "nucleus" is meant a membrane-bounded organelle containing most or all of the DNA of a cell. The DNA is packaged into chromosomes in a decondensed form. Preferably, the membrane encapsulating the DNA includes one or two lipid bilayers or has nucleoporins.

By "nucleus that has less than four sets of homologous chromosomes" is meant a nucleus that has a DNA content of less than 4n, where "n" is the number of chromosomes found in the normal haploid chromosome set of a mammal of a particular genus or species. Such a nucleus does not have four copies of each gene or genetic locus. For example, the nucleus is diploid and thus has two sets of homologous chromosomes.

By "enrichment or depletion of a factor" in an extract is meant the addition or removal of a naturally-occurring or recombinant factor by at least 20% of the amount of the factor originally present in an extract. Alternatively, a naturally occurring or recombinant factor that is not naturally present in the extract may be added. Suitable factors include proteins such as DNA methyltransferases, histone deacetylases, histones, nuclear lamins, transcription factors, activators, and repressors; membrane vesicles, and organelles. In one embodiment, the factor is purified prior to being added to the extract, as described below. Alternatively, one of the purification methods described below may be used to remove an undesired factor from the extract.

By "purified" is meant a factor that is separated from other components that naturally accompany it. A factor is substantially pure when it is at least 50%, by weight, free from proteins, antibodies, and naturally-occurring organic molecules with which it is naturally associated. In certain embodiments, the factor is at least 75%, 90%, or 99%, by weight, pure. A substantially pure factor may be obtained by chemical synthesis, separation of the factor from natural sources, or production of the factor in a recombinant host cell that does not naturally produce the factor. Proteins, vesicles, and organelles may be purified by one skilled in the art using

standard techniques such as those described by Ausubel et al., (Current Protocols in Molecular Biology, John Wiley & Sons, New York, 2000). The factor can be at least 2, 5, or 10 times as pure as the starting material, as measured using polyacrylamide gel electrophoresis, column chromatography, optical density, HPLC analysis, or western analysis (Ausubel et al., *supra*). Suitable methods of purification include immunoprecipitation, column chromatography such as immunoaffinity chromatography, magnetic bead immunoaffinity purification, and panning with a plate-bound antibody.

By "recloned" is meant used in a second round of cloning. In particular, a cell from an embryo, fetus, or adult generated from the methods of the invention may be incubated in a mitotic extract to form condensed chromatin for insertion into an enucleated oocyte, as described for the first round of cloning. Performing two or more rounds of cloning may result in additional reprogramming of the donor chromatin, thereby increasing the chance of generating a viable offspring after the last round of cloning.

By "viable offspring" is meant a mammal that survives *ex utero*. The mammal must survive for at least one second, but will preferably survive one minute, one hour, one day, one week one month, six months, or one year from the time it exits the maternal host. The mammal does not require the circulatory system of an *in utero* environment for survival.

By "embryo" or "embryonic" is meant a developing cell mass that has not implanted into the uterine membrane of a maternal host. Hence, the term "embryo" may refer to a fertilized oocyte, an oocyte containing donor condensed chromatin or nucleus, a pre-blastocyst stage developing cell mass, or any other developing cell mass that is at a stage of development prior to implantation into the uterine membrane of a maternal host and prior to formation of a genital ridge. An embryo may represent multiple stages of cell development. For example, a one cell embryo can be referred to as a zygote; a solid spherical mass of cells resulting from a cleaved embryo can be referred to as a morula, and an embryo having a blastocoel can be referred to as a blastocyst. An "embryonic cell" is a cell isolated from or contained in an embryo.

By "fetus" is meant a developing cell mass that has implanted into the uterine membrane of a maternal host. A fetus may have defining features such as a genital ridge, which is easily identified by a person of ordinary skill in the art. A "fetal cell" is any cell isolated from or contained in a fetus.

By "mutation" is meant an alteration in a naturally-occurring or reference nucleic acid sequence, such as an insertion, deletion, frameshift mutation, silent mutation, nonsense mutation, or missense mutation. The amino acid sequence

encoded by the nucleic acid sequence can have at least one amino acid alteration from a naturally-occurring sequence. Examples of recombinant DNA techniques for altering the genomic sequence of a cell, embryo, fetus, or mammal include inserting a DNA sequence from another organism (e.g., a human) into the genome, deleting one or more DNA sequences, and introducing one or more base mutations (e.g., site-directed or random mutations) into a target DNA sequence. Examples of methods for producing these modifications include retroviral insertion, artificial chromosome techniques, gene insertion, random insertion with tissue specific promoters, homologous recombination, gene targeting, transposable elements, and any other method for introducing foreign DNA. All of these techniques are well known to those skilled in the art of molecular biology (see, for example, Ausubel et al., *supra*). Condensed chromatin and nuclei from transgenic cells containing modified DNA may be used in the methods of the invention.

By "donor cell" is meant a cell from which a nucleus or chromatin is derived, or a cell whose plasma membrane is removed, permeabilized, or breached for subsequent remodeling.

The term "embryonic stem cells" refers to cells isolated from the inner cell mass of blastocysts or morulae and that have been serially passaged as cell lines.

The term "human embryonic stem cells" (hES cells) refers to cells isolated from the inner cell mass of human blastocysts or morulae and that have been serially passaged as cell lines.

The term "human embryo-derived cells" refers to morula-derived cells, blastocyst-derived cells including those of the inner cell mass, embryonic shield, or epiblast, or other totipotent or pluripotent stem cells of the early embryo, including primitive endoderm, ectoderm, and mesoderm and their derivatives, but excluding human embryonic stem cells that have been passaged as cell lines.

By "permeabilization" is meant the formation of pores in the plasma membrane or the partial or complete removal of the plasma membrane such that reprogramming media, or enhanced recombination media, can contact the nuclear genome.

By a "permeabilized cell" is meant a cell that has undergone permeabilization.

By "reprogramming media" is meant a solution that allows the removal of a factor from a cell, nucleus, condensed chromatin, or chromosome or the addition of a factor from the solution to the cell, nucleus, condensed chromatin, or chromosome. The addition or removal of a factor increases or decreases the level of expression of an mRNA or protein in the donor cell, condensed chromatin, or nucleus or in a cell containing the reprogrammed condensed chromatin or nucleus.

By "fusigenic compound" is meant a compound that increases the probability that condensed chromatin or a nucleus is inserted into a recipient cell when located adjacent to the cell. For example, the fusigenic compound may increase the affinity of a condensed chromatin or a nucleus for the plasma membrane of a cell. The fusigenic compound may also promote the joining of the nuclear membrane of a nucleus with the plasma membrane of a cell.

By "substantially identical" is meant having a sequence that is at least 60% identical to that of another sequence. Sequence identity is measured using sequence analysis software with the default parameters specified therein (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

Methods and Examples

Bulk preparation of donor nuclei for use in cloning

As many as several million nuclei may be isolated from synchronized or unsynchronized cell populations in culture or cells isolated directly from tissue in vivo. The cell populations may be synchronized naturally or chemically. Preferably, at least 40, 60, 80, 90, or 100% of the cells in a population are arrested in G_0 or G_1 phase. To accomplish this, cells may be incubated, for example, in low serum, such as 5%, 2%, or 0% serum, for 1, 2, 3, or more days to increase the percentage of cells in G_0, phase. To synchronize cells in G_1, the cells may be grown to confluence as attached cells and then incubated in 0.5-1 μg/ml nocodazole (Sigma Chemicals, St. Louis, MO) for 17-20 hours, as described previously (see, for example, Collas et al., 1999 and references therein). The flasks containing the attached cells are shaken vigorously by repeatedly tapping the flasks with one hand, resulting in the detachment of mitotic cells and G_1, phase doublets. The G_1 phase doublets are pairs of elongated cells at the end of the division process that are still connected by a thin bridge. Detached G_1 phase doublets may be isolated from the media based on this characteristic doublet structure. The G_1 phase doublets may remain attached or may divide into two separate cells after isolation.

The synchronized or unsynchronized cells are harvested in phosphate buffered saline (PBS) using standard procedures, and several washing steps are performed to transfer the cells from their original media into a hypotonic buffer (10 mM HEPES, pH, 7.5, 2 mM $MgCl_2$, 25 mM KCl, 1 mM DTT,10 μM aprotinin, 10 μM leupeptin, 10 μM pepstatin A, 10 μM soybean trypsin inhibitor, and 100 μM

PMSF). For example, the cells may be washed with 50 ml of PBS and pelleted by centrifugation at 500 x g for 10 minutes 4°C. The PBS supernatant is decanted, and the pelleted cells are resuspended in 50 ml of PBS and centrifuged, as described above. After this centrifugation, the pelleted cells are resuspended in 20-50 volumes of ice-cold hypotonic buffer and centrifuged at 500 x g for 10 minutes at 4°C. The supernatant is again discarded and approximately 20 volumes of hypotonic buffer are added to the cell pellet. The cells are carefully resuspended in this buffer and incubated on ice for at least one hour, resulting in the gradual swelling of the cells.

To allow isolation of the nuclei from the cells, the cells are lysed using standard procedures. For example, 2-5 ml of the cell suspension may be transferred to a glass homogenizer and Dounce homogenized using an initial 10-20 strokes of a tight-fitting pestle. Alternatively, the cell suspension is homogenized using a motorized mixer (e.g., Ultraturrax). If desired, cell lysis may be monitored using phase contrast microscopy at 40-fold magnification. During this homogenization, the nuclei should remain intact and preferably most or all of the originally attached cytoplasmic components such as vesicles, organelles, and proteins should be released from the nuclei. If necessary, 1-20 mg/ml of the cytoskeletal inhibitors, cytochalasin B or cytochalasin D, may be added to the aforementioned hypotonic buffer to facilitate this process. Homogenization is continued as long as necessary to lyse the cells and release cytoplasmic components from the nuclei. For some cell types, as many as 100, 150, or more strokes may be required.

The lysate is then transferred into a 15 ml conical tube on ice, and the cell lysis procedure is repeated with the remainder of the suspension of swollen cells. Sucrose from a 2 M stock solution made in hypotonic buffer is added to the cell lysate, resulting in a final concentration of 250 mM sucrose. This solution is mixed by inversion, and the nuclei are pelleted by centrifugation at 400 x g in a swing out rotor for 10 to 40 minutes at 4°C. The supernatant is then discarded, and the pelleted nuclei are resuspended in 10-20 volumes of nuclear buffer (10 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 250 mM sucrose, 25 mM KCl, 1 mM DTT, 10 μM aprotinin, 10 μM leupeptin, 10 μM pepstatin A, 10 μM soybean trypsin inhibitor, and 100 μM PMSF). The nuclei are sedimented and resuspended in 1-2 volumes of nuclear buffer, as described above. The freshly isolated nuclei may either be used immediately for *in vitro* reprogramming and nuclear transfer as described herein or stored for later use.

For storage, the nuclei are diluted in nuclear buffer to a concentration of approximately 10^6 ml. Glycerol (2.4 volumes of 100% glycerol) is added and mixed well by gentle pipetting. The suspension is aliquoted into 100-500 μl volumes in 1.5-ml tubes on ice, immediately frozen in a methanol-dry ice bath, and stored at -

80°C. Prior to use, aliquots of the nuclei are thawed on ice or at room temperature. One volume of ice-cold nuclear buffer is added, and the solution is centrifuged at 1,000 x g for 15 minutes in a swing out rotor. The pelleted nuclei are resuspended in 100-500 μl nuclear buffer and centrifuged as described above. The pelleted nuclei are then resuspended in a minimal volume of nuclear buffer and stored on ice until use.

Preparation of mitotic extract for use in reprogramming donor genetic material

For the preparation of a mitotic extract, a somatic cell line (e.g., of fibroblasts) is synchronized in mitosis by incubation in 0.5-1.0 μg/ml nocodazole for 17-20 hours and the mitotic cells are detached by vigorous shaking, as described above. The detached G_1 phase doublets may be discarded, or they may be allowed to remain with the mitotic cells which constitute the majority off the detached cells (typically at least 80%). The harvested detached cells are centrifuged at 500 x g for 10 minutes in a 10 ml conical tube at 4°C. Several cell pellets are pooled, resuspended in a total volume of 50 ml of cold PBS, and centrifuged at 500 x g for 10 minutes at 4°C. This PBS washing step is repeated. The cell pellet is resuspended in approximately 20 volumes of ice-cold cell lysis buffer (20 mM HEPES, pH 8.2, 5 mM $MgCl_2$, 10 mM EDTA, 1 mM DTT, 10 μM aprotinin, 10 μM leupeptin, 10 μM pepstatin A, 10 μM soybean trypsin inhibitor, 100 μM PMSF, and optionally 20 μg/ml cytochalasin B), and the cells are sedimented by centrifugation at 800 x g for 10 minutes at 4°C. The supernatant is discarded, and the cell pellet is carefully resuspended in no more than one volume of cell lysis buffer.

The cells are incubated on ice for one hour to allow swelling of the cells. The cells are lysed by either sonication using a tip sonicator or Dounce homogenization using a glass mortar and pestle. Cell lysis is performed until at least 90% of the cells and nuclei are lysed, which may be assessed using phase contrast microscopy. The sonication time required to lyse at least 90% of the cells and nuclei may vary depending on the type of cell used to prepare the extract.

The cell lysate is placed in a 1.5-ml centrifuge tube and centrifuged at 10,000 to 15,000 x g for 15 minutes at 4°C using a table top centrifuge. The tubes are removed from the centrifuge and immediately placed on ice. The supernatant is carefully collected using a 200 μl pipette tip, and the supernatant from several tubes is pooled and placed on ice. This supernatant is the "mitotic cytoplasmic" or "MS 15" extract. This cell extract may be aliquoted into 50 μl or 10 μl volumes of extract per tube on ice, depending on whether the regular or micromethod for generation of condensed chromatin will be used. The extracts are immediately flash-frozen on liquid nitrogen and stored at -80°C until use. Alternatively, the cell extract is placed

in an ultracentrifuge tube on ice (e.g., fitted for an SW55 Ti rotor; Beckman). If necessary, the tube is overlayed with mineral oil to the top. The extract is centrifuged at 200,000 x g for three hours at 4°C to sediment membrane vesicles contained in the MS15 extract. At the end of centrifugation, the oil is discarded. The supernatant is carefully collected, pooled if necessary, and placed in a cold 1.5 ml tube on ice. This supernatant is referred to as "MS200" or "mitotic cytosolic" extract. The extract is aliquoted and frozen as described for the MS 15 extract.

Formation of condensed chromatin by exposure of nuclei to a mitotic extract

An aliquot of MS15 or MS200 extract is thawed on ice. An ATP-generating system (0.6 μl) is added to 20 μl of extract and mixed by vortexing. For the preparation of the ATP -generating system, equal proportions of mM 100 ATP stock, 1 M creatine phosphate, and 2.5mg/ml creatine kinase stock solutions (100x) made in H_2O are mixed and stored on ice until use. After addition of the ATP generating system to the extract, the final concentrations are 1 mM ATP, 10 mM creatine phosphate, and 25 μg/ml creatine kinase.

The nuclei suspension is added to the extract at a concentration of 1 μl nuclei per 10 μl of extract, mixed well by pipeting, and incubated in 30, 33, 35, 37, or 39°C water bath. The tube containing the mixture is tapped gently at regular intervals to prevent chromosomes from clumping at the bottom of the tube. Nuclear envelope breakdown and chromosome condensation is monitored at regular intervals, such as every 15 minutes, under a microscope. When the nuclear envelope has broken down and chromosomes have started to condense, the procedure for recovery of condensed chromatin from the extract is started.

Formation of decondensed chromatin by exposure of nuclei to a mitotic extract and anti-NuMA antibodies

Alternatively, chromatin that is not condensed or only partially condensed may be formed by performing the above procedure after pre-loading the isolated nuclei with an antibody to the nuclear NuMA (Steen et al., J. Cell Biol. 149, 531-536, 2000). This procedure allows the removal of nuclear components from chromatin by the dissolution of the nuclear membrane surrounding the donor nuclei; however, the condensation step is inhibited by addition of the anti-NuMA antibody. Preventing chromosome condensation may reduce the risk of chromosome breakage or loss while the chromosomes are incubated in the mitotic extract.

For this procedure, purified cell nuclei (2,000 nuclei/μl) are permeabilized in 500 μl nuclear buffer containing 0.75 μg/ml lysolecithin for 15 minutes at room temperature. Excess lysolecithin is quenched by adding 1 ml of 3% BSA made in

nuclear buffer and incubating for 5 minutes on ice. The nuclei are then sedimented and washed once in nuclear buffer. The nuclei are resuspended at 2,000 nuclei/μl in 100 μl nuclear buffer containing an anti-NuMA antibody (1:40 dilution; Transduction Laboratories). After an one-hour incubation on ice with gentle agitation, the nuclei are sedimented at 500 x g through 1 M sucrose for 20 minutes. The nuclei are then resuspended in nuclear buffer and added to a mitotic extract containing an ATP regenerating system, as described in the previous section. Optionally, the anti-NuMA antibody may be added to the extract to further prevent chromosome condensation.

Formation of decondensed chromatin by exposure of nuclei to a detergent and/or salt solution or to a protein kinase solution

Chromatin that is not condensed or only partially condensed may also be formed by exposure to a detergent or protein kinase. Detergent may be used to solubilize nuclear components that are either unbound or loosely bound to the chromosomes in the nucleus, resulting in the removal of the nuclear envelope. For this procedure, purified cell nuclei (2,000-10,000 nuclei/μl) are incubated in nuclear buffer supplemented with a detergent, such as 0.1% to 0.5% Triton X-100 or NP-40. To facilitate removal of the nuclear envelope, additional salt, such as NaCl, may be added to the buffer at a concentration of approximately 0.1, 0.15, 0.25, 0.5, 0.75, or 1 M. After a 30-60 minute incubation on ice with gentle shaking, the nuclei are sedimented by centrifugation at 1,000 x g in a swing-out rotor for 10-30 minutes, depending on the total volume. The pelleted nuclei are resuspended in 0.5 to 1 ml nuclear buffer and sedimented as described above. This washing procedure is repeated twice to ensure complete removal of the detergent and extra salt.

Alternatively, the nuclear envelope may be removed using recombinant or naturally-occurring protein kinases, alone or in combination. Preferably, the protein kinases are purified using standard procedures or obtained in purified form from commercial sources. These kinases may phosphorylate components of the nuclear membrane, nuclear matrix, or chromatin, resulting in removal of the nuclear envelope (see, for example, Collas and Courvalin, Trends Cell Biol. 10:5-8, 2000). Preferred kinases include cyclin-dependent kinase 1 (CDK1), protein kinase C (PKC), protein kinase A (PKA), MAP kinase, and calcium/calmodulin dependent kinase (CamKII). For this method, approximately 20,000 purified nuclei are incubated in 20 μl of phosphorylation buffer at room temperature in a 1.5 ml centrifuge tube. A preferred phosphorylation buffer for CDK1 (Upstate Biotechnology) contains 200 mM NaCl, 50 mM Tris-HCl (pH 7.2-7.6), 10 mM $MgSO_4$, 80 mM α-glycerophosphate, 5 mM EGTA, 100 μM ATP, and 1 mM DTT.

For PKC, a preferred buffer contains 200 mM NaCl, 50 mM Tris-HCl (pH 7.2-7.6), 10 mM $MgSO_4$, 100 μM $CaCl_2$, 40 μg/ml phosphatidylserine, 20 μM diacylglycerol, 100 μM ATP, and 1 mM DTT. If both PKC and CDKl are used simultaneously, CDKl the phosphorylation buffer supplemented with 40 μg/ml phosphatidylserine and 20 μM diacylglycerol is used. A preferred phosphorylation buffer for PKA includes 200 mM NaCl, 10 mM MgSO4, 10 mM Tris, pH 7.0, 1 mM EDTA, and 100 μM ATP. For MAP kinase, the PKA phosphorylation buffer supplemented with 10 mM $CaCl_2$ and 1 mM DTT may be used. For CamKII, either PKA buffer supplemented with 1 mM DTT or a Cam Kinase assay kit from Upstate Biotechnology (Venema et al. J. Biol. Chem 272:28187-190, 1997) is used.

The phosphorylation reaction is initiated by adding a protein kinase to a final amount of 25-100 ng. The reaction is incubated at room temperature for up to one hour. Nuclear envelope breakdown may be monitored by microscopy during this incubation, such as at 15 minute intervals. After nuclear envelope breakdown, nuclei are washed three times, as described above for the removal of the detergent solution.

Recovery of condensed chromatin from the extract, detergent, and salt solution, or protein kinase solution

The extract or solution containing the condensed, partially condensed, or not condensed chromatin is placed under an equal volume of 1 M sucrose solution made in nuclear buffer. The chromatin is sedimented by centrifugation at 1,000 x g for 10-30 minutes depending on the sample volume in a swing out rotor at 4°C. The supernatant is discarded and the pelleted chromatin is carefully resuspended by pipetting in 0.1-1.0 ml nuclear buffer or lipofusion buffer (150 mM NaCl, 10 μM aprotinin, 10 μM leupeptin, 10 μM pepstatin A, 10 μM soybean trypsin inhibitor, and 100 μM PMSF in either 20 mM HEPES around pH 7.0 or pH 7.5 or 20 mM MES around pH 6.2) and centrifuged at 1,000 x g for 10-30 minutes. The supernatant is discarded, and the pelleted chromatin is resuspended in nuclear buffer or lipofusion buffer and stored on ice until use. Each resuspended chromatin pellet is transferred to a 20 μl drop of HEPES buffered medium under oil in a micromanipulation dish. One resuspended chromatin pellet is inserted into each enucleated oocyte, as described below.

Micromethod for preparation of condensed chromatin

A 10-20 μl drop of MS15 or MS200 extract containing an ATP generating system, a detergent and salt solution, or a protein kinase solution as described above is placed in a petri dish. A 50-μl drop of isolated G_1 phase cell doublets or G_0 phase

cells in culture medium, a separate 50 μl "lysis" drop of HEPES-or bicarbonate-buffered medium containing 0.1% Triton X-100 or NP-40 for use in facilitating cell lysis, and a 50 μl drop of oocyte injection medium is then added. Each of these drops is covered with CO_2 equilibrated mineral oil. 50 μl "wash drop" of culture medium is also added to the petri dish for use in washing the lysed cells or nuclei.

Cells are transferred to the lysis drop using a micropipette. The cell membranes are lysed in the pipette by gentle repeated aspirations. When the cell is lysed, the lysate is gently expelled into the wash drop, and the nucleus is immediately reaspirated to remove detergent. Optionally, the nuclei may be permeabilized and incubated with anti-NuMA antibodies prior to being added to the mitotic extract. The nucleus is then expelled into the drop of MS15, MS200, detergent and salt solution, or protein kinase solution. Nuclear breakdown and chromosome condensation is monitored as described above. Once the nuclear envelope has broken down and, if a mitotic extract without anti-NuMA antibodies was used, the chromosomes have started to condense, a single intact mass of condensed chromatin is isolated with a micropipette and transferred to an enucleated recipient oocyte, as described below.

Enucleation of oocytes

The recipient oocyte can be a metaphase II stage oocyte. At this stage, the oocyte may be activated or is already sufficiently activated to treat the introduced condensed chromatin as it does a fertilizing sperm. For enucleatation of the oocyte, part or preferably all of the DNA in the oocyte is removed or inactivated. This destruction or removal of the DNA in the recipient oocyte prevents the genetic material of the oocyte from contributing to the growth and development of the cloned mammal. One method for destroying the pronucleus of the oocyte is exposure to ultraviolet light (Gurdon, in Methods in Cell Biology, Xenopus Laevis: Practical Uses in cell and Molecular Biology, Kay and Peng, eds,, Academic Press, California, volume 36: pages 299-309, 1991).

Alternatively, the oocyte pronucleus may be surgically removed by any standard technique (see, for example, McGrath and Solter, Science 220: 1300-1319, 1983). In one possible method, a needle is placed into the oocyte, and the nucleus is aspirated into the inner space of the needle. The needle may then be removed from the oocyte without rupturing the plasma membrane (U. S. Patent Numbers 4,994,384 and 5,057,420).

Lipofusion for insertion of condensed chromatin into oocytes

Chromatin may be introduced into recipient oocytes by lipofusion as described below or by standard microinjection or electrofusion techniques (see, for example, U. S. Patent Numbers 4,997,384 and 5,945,577). The following lipofusion method may also be used in other applications to insert chromosomes into other recipient cells.

Condensed chromatin is isolated from the mitotic extract, detergent and salt solution, or protein kinase solution by centrifugation, and then washed with lipofusion buffer, as described above. The condensed chromatin may be in stored in ice-cold lipofusion buffer until use. Alternatively, the condensed chromatin is aliquoted, frozen in liquid nitrogen or in a methanol-dry ice bath, and stored frozen at -80°C. The lipofusion solution is prepared by mixing one or more fusigenic reagents with the lipofusion buffer in respective proportions ranging from 5:1 to 1:10 approximately. The fusigenic reagents consist of, but are not limited to, polyethylene glycol (PEG) and lipophilic compounds such as Lipofectin®, Lipofectamin®, DOTAP® DOSPA®, DOPE®, and membrane vesicle fractions. For example, a cationic lipid, such as DOTAP®, may be used at a concentration of approximately 0.1 to 30 μg/ml in lipofusion buffer. Alternatively, a liposome formulation consisting of a mixture of a cationic lipid and 3 neutral lipid, such as DOPE®, may be used.

The condensed chromatin, either freshly prepared or frozen and thawed, is mixed with the lipofusion solution to allow coating of the condensed chromatin with the compound. Incubation takes place at a temperature of 20-30°C for a period of approximately 10-30 minutes. Microdrops containing the condensed chromatin in the lipofusion solution are placed under CO_2 equilibrated mineral oil. A drop containing the enucleated recipient oocytes is also prepared. The condensed chromatin coated with the lipofusion reagent is picked up in a micropipette and inserted in the perivitellin space, between the oocyte cytoplasm and the zona pellucida. The condensed chromatin is placed next to the oocyte membrane to ensure contact with the oocyte. The condensed chromatin-oocyte complexes are maintained at a temperature of 20-30°C, and fusion is monitored under the microscope. Once fusion has occurred, reconstituted oocytes are activated as described below.

Activation, culturing, and transplantation of reconstituted oocytes

To prevent polar body extrusion and chromosome loss, the oocyte may be activated in the presence of cytochalasin B, or cytochalasin B may be added immediately after activation (Wakayama et al., PNAS 96:14984-14989, 1999;

Wakayama et al., Nature Genetics 24:108-109, 2000). Either electrical or non-electrical means may be used for activating reconstituted oocytes. Electrical techniques for activating cells are well known in the art (see, for example, U. S. Patent Numbers 4,994,384 and 5,057,420). Non-electrical means for activating cells may include any method known in the art that increases the probability of cell division. Examples of non-electrical means for activating an oocyte include incubating the oocyte in the presence of ethanol; inositol trisphosphate; Ca^{++} ionophore and a protein kinase inhibitor; a protein synthesis inhibitor; phorbol esters; thapsigargin, or any component of sperm. Other non-electrical methods for activation include subjecting the oocyte to cold shock or mechanical stress.

Alternatively, one to three hours after nuclear transfer, oocytes may be incubated for approximately six hours in medium containing Sr^{2+} to activate them and cytochalasin B to prevent cytokinesis and polar body extrusion (Wakayama et al., PNAS 96:14984-14989, 1999; Wakayama et al., Nature Genetics 24:108-109, 2000). Depending on the type of mammal cloned, the preferred length of activation may vary. For example, in domestic animals such as cattle, the oocyte activation period generally ranges from about 16-52 hours or preferably about 28-42 hours.

After activation, the oocyte is placed in culture medium for an appropriate amount of time to allow development of the resulting embryo. At the two-cell stage or a later stage, the embryo is transferred into a foster recipient female for development to term. For bovine species, the embryos are typically cultured to the blastocyst stage (e.g., for approximately 6-8 days) before being transferred to maternal hosts. For other cloned animals, an appropriate length for in vitro culturing is known by one skilled in the art or may be determined by routine experimentation.

Methods for implanting embryos into the uterus of a mammal are also well known in the art. Preferably, the developmental stage of the embryo is correlated with the estrus cycle of the host mammal. Once the embryo is placed in the uterus of the mammal, the embryo may develop to term. Alternatively, the embryo is allowed to develop in the uterus until a chosen time, and then the embryo (or fetus) is removed using standard surgical methods to determine its health and viability. Embryos from one species may be placed into the uterine environment of an animal from another species. For example, bovine embryos can develop in the oviducts of sheep (Stice and Keefer, Biology of Reproduction 48:715-719, 1993). Any cross-species relationship between embryo and uterus may be used in the methods of the invention.

Lipofusion of nuclei with oocytes or other recipient cells

The lipofusion solution is prepared by mixing one or more fusigenic reagents with lipofusion buffer in respective proportions ranging from approximately 5:1 to 1:10, as described above. Nuclei, either freshly prepared or frozen and thawed as described above, are mixed with the lipofusion solution to allow coating of the nuclei with the compound. Incubation takes place at a temperature of 20-30°C for a period of approximately 10-30 minutes. Microdrops containing nuclei in the lipo1usion solution are placed under CO_2 equilibrated mineral oil. A drop containing the recipient cell, preferably an enucleated cell, is also prepared. Enucleated recipient cells are prepared by physically removing the chromosomes or the nucleus by micromanipulation or by damaging the genetic material by exposure to UV light, as described above. For insertion into oocytes, the nuclei coated with the lipofusion reagent are picked up in a micropipette and inserted in the periviteliin space, between the oocyte cytoplasm and the zona pellucida. For insertion into other recipient cells, the coated nuclei are preferably placed next to the cell membrane to ensure contact with the cell. The nucleus-cell complexes are maintained at a temperature of 20-30°C, and fusion is monitored using a microscope. Once fusion has occurred, reconstituted oocytes are activated as described above.

Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

WE CLAIM:

1. A method of cloning a mammal, said method comprising:

(a) contacting a donor nucleus with a mitotic extract, detergent, and salt solution, or protein kinase solution under conditions that allow formation of condensed chromatin without causing DNA replication;

(b) inserting said condensed chromatin into an enucleated oocyte; and

(c) transferring said oocyte or an embryo formed from said oocyte into the uterus of a host mammal under conditions that allow said oocyte or said embryo to develop into a fetus.

2. The method of claim 1, wherein said condensed chromatin from (a) is purified from said extract prior to insertion into said enucleated oocyte.

3. The method of claim 1, wherein said fetus develops into a viable offspring.

4. The method of claim 1, wherein said oocyte from (b) is cultured under conditions that allow cell division and one of the resulting cells is recloned one or more times.

5. The method of claim 1, wherein said donor nucleus and said oocyte are from the same species.

6. The method of claim 1, wherein said mammal is a non-human mammal.

7. The method of claim 6, wherein said non-human mammal is a cow, sheep, rabbit, pig, mouse, rat, goat, cat, dog, or buffalo.

8. The method of claim 6, wherein said non-human mammal is a dog.

9. The method of claim 6, wherein said non-human mammal is a cat.

10. The method of claim 6, wherein said non-human mammal is a cow.

11. The method of claim 1, wherein said donor nucleus is dipioid.

12. The method of claim 1, wherein said donor nucleus is from an adult, fetal, or embryonic cell.

13. The method of claim 1, wherein said donor nucleus is from an epithelial cell, neural cell, epidermal cell, keratinocyte, hematopoietic cell, melanocyte, chondrocyte, B-lymphocyte, T-lymphocyte, erythrocyte, macrophage, monocyte, fibroblast, muscle cell, embryonic stem cell, or embryonic germ cell.

14. The method of claim 1, wherein said donor nucleus is from a cell of the female reproductive system.

15. The method of claim 14, wherein said cell of the female reproductive system is a mammary gland, ovarian cumulus, grandulosa, or oviductal cell.

16. The method of claim 1, wherein said (b) comprises contacting said condensed chromatin and said oocyte with a fusigenic compound under conditions that allow said condensed chromatin to enter said oocyte.

17. A method of inserting chromosomes into a recipient cell, said method comprising contacting said chromosomes and said cell with a fusigenic compound under conditions that allow said chromosomes to enter said recipient cell.

18. The method of claim 17, wherein said chromosomes are incubated with said fusigenic compound prior to being contacted with said recipient cell.

19. The method of claim 17, wherein said chromosomes are condensed.

20. The method of claim 17, wherein said chromosomes and said recipient cell are from the same species.

21. The method of claim 17, wherein said recipient cell is an oocyte.

22. The method of claim 17, wherein said recipient cell or said chromosomes are from a non-human mammal.

23. The method of claim 22, wherein said non-human mammal is a cow, sheep, rabbit, pig, mouse, rat, goat, cat, dog, or buffalo.

24. The method of claim 22, wherein said non-human mammal is a dog.

25. The method of claim 22, wherein said non-human mammal is a cat.

26. The method of claim 22, wherein said non-human mammal is a cow.

27. The method of claim 17, wherein said recipient cell is an adult, fetal, or embryonic cell or said chromosomes are from an adult, fetal, or embryonic cell.

28. The method of claim 17, wherein said chromosomes are from a donor cell and all of said chromosomes of said donor cell are inserted into said recipient cell.

29. The method of claim 17, wherein said chromosomes are obtained by contacting a donor nucleus with a mitotic extract, detergent, and salt solution, or protein kinase solution under conditions that allow formation of a condensed chromatin without causing DNA replication.

30. The method of claim 17, wherein said fusigenic compound is polyethylene glycol, Lipofectin®, Lipofectinamin®, DOTAP®, DOSPA® or DOPE®.

31. A method of inserting a nucleus into a recipient cell, said method comprising contacting said nucleus and said cell with a fusigenic compound under conditions that allow said nucleus to enter said recipient cell, wherein said fusigenic compound is not a polymer.

32. The method of claim 31, wherein said nucleus is incubated with said fusigenic compound prior to being contacted with said recipient cell.

33. The method of claim 31, wherein said nucleus and said recipient cell are from the same species.

34. The method of claim 31, wherein said recipient cell is an oocyte.

35. The method of claim 31, wherein said recipient cell or said nucleus are from a non-human mammal.

36. The method of claim 35, wherein said non-human mammal is a cow, sheep, rabbit, pig, mouse, rat, goat, cat, dog, or buffalo.

37. The method of claim 35, wherein said non-human mammal is a dog.

38. The method of claim 35, wherein said non-human mammal is a cat.

39. The method of claim 35, wherein said non-human mammal is a cow.

40. The method of claim 31, wherein said recipient cell is an adult, fetal, or embryonic cell or said nucleus is from an adult, fetal, or embryonic cell.

41. The method of claim 31, wherein said fusigenic compound is a lipid.

42. The method of claim 31, wherein said fusigenic compound is Lipofectin®, Lipofectamin®, DOTAP®, DOSPA®, or DOPE®.

43. A method of reprogramming the nucleus of a somatic cell, the method compromising contacting said nucleus with cytoplasmic extract.

44. The method of claim 43, wherein said cytoplasmic extract is from metaphase cells.

45. The method of claim 43, wherein the somatic cell is a fibroblast.

46. The method of claim 43, wherein said nucleus is isolated from said somatic cell and incubated in a cytoplasmic extract.

47. The method of claim 43, wherein said reprogrammed nucleus is injected or transplanted into a recipient cell.

48. The method of claim 43, wherein said recipient cell is an oocyte.

49. The method of claim 43, wherein said reprogramming results in condensed chromatin.

50. The method of claim 43, wherein said chromatin is injected into a recipient cell.

51. The method of claim 50, wherein said recipient cell is an enucleated oocyte.

52. A method of cloning a non-human mammal, said method comprising:

(a) incubating a permeabilized cell in a reprogramming media under conditions that allow the removal of a factor from a nucleus, chromatin mass, or chromosome of said permeabilized cell or the addition of a factor from said reprogramming media to said nucleus, chromatin mass, or chromosome, thereby forming a reprogrammed cell;

(b) inserting said reprogrammed cell into a nucleated or enucleated oocyte, thereby forming a nuclear transfer oocyte; and

(c) transferring said nuclear transfer oocyte or an embryo formed from said nuclear transfer oocyte into the uterus of a host mammal under conditions that allow said nuclear transfer oocyte or said embryo to develop into a fetus.

53. The method of claim 52, wherein said reprogramming media comprises a cell extract.

54. The method of claim 52, wherein the nucleus of said permeabilized cellremains membrane-bounded and the chromatin in said nucleus does not condense during incubation in said reprogramming media.

55. The method of claim 52, wherein a chromatin mass is formed from incubation of said permeabilized cell in said reprogramming media.

56. The method of claim 52, wherein said reprogrammed cell is incubated under conditions that allow the membrane of said reprogrammed cell to reseal.

57. The method of claim 52, wherein said reprogrammed cell is purified from said reprogramming media prior to insertion into said nuclear transfer oocyte.

58. The method of claim 52, wherein said fetus develops into a viable offspring.

59. The method of claim 52, wherein said nuclear transfer oocyte from (b) is cultured under conditions that allow cell division and one of the resulting cells is recloned one or more times.

60. The method of claim 52, wherein said permeabilized cell and said nuclear transfer oocyte are from the same species.

61. The method of claim 52, wherein said non-human mammal is a cow, sheep, rabbit, pig, mouse, rat, goat, cat, dog or buffalo.

62. The method of claim 52, wherein said non-human mammal is a dog.

63. The method of claim 52, wherein said non-human mammal is a cat.

64. The method of claim 52, wherein said non-human mammal is a cow.

65. The method of claim 52, wherein said permeabilized cell is a fibroblast, epithelial cell, neural cell, epidermal cell, keratinocyte, hematopoietic cell, melanocyte, chondrocyte, B-lymphocyte, T-lymphocyte, erythrocyte, macrophage, monocyte, muscle cell, embryonic stem cell, embryonic germ cell, fetal cell, placental cell, or embryonic cell.

66. The method of claim 52, wherein said permeabilized cell is a cell of the female reproductive system.

67. The method of claim 66, wherein said permeabilized cell is a mammary gland, ovarian cumulus, grandulosa, or oviductal cell.

68. The method of claim 52, wherein said nuclear transfer ooctye from (b) expresses lamin A, lamin C, or NuMA protein at a level that is less than 5 fold greater than the corresponding level expressed by a control oocyte from the same species.

69. A method of cloning mammalian pluripotent stem cells, said method comprising:

a. contacting a donor nucleus with a mitotic extract, detergent and salt solution, or protein kinase solution under conditions that allow formation of a chromatin mass without causing DNA replication;
b. inserting said chromatin mass into an enucleated oocyte; and
c. incubating the oocyte or an embryo formed from said oocyte until a morula or blastocyst is obtained; and

d. obtaining from said embryo pluripotent stem cells.

70. The method of claim 69, wherein said donor nucleus is from a human cell.

71. The method of claim 69, wherein said donor nucleus is from a cat or dog cell.

72. The method of claim 69, wherein said pluripotent stem cells are embryonic stem cells.

73. The method of claim 69, wherein said pluripotent stem cells are embryo-derived cells.